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14. ABSTRACT
The purpose of this research is to determine mechanisms through which hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), environmental degradation product of high energetic munition hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), causes persistent anemia in the rat. We have hypothesized MNX targets hematopoeitic stem cells and, like other myelosuppressive chemicals, will be fibrogenic to the bone marrow. Findings of this period are: 1) the inability of RDX and MNX to directly oxidize hemoglobin ferrous iron to methemoglobin in vitro and of MNX to produce methemoglobin in MNX-treated rats, an alternative mechanism for the observed anemia and 2) nitramine targeting of an early multipotential bone marrow stem cell at earlier times after exposure (7d) and flow cytometric assessment of myeloid and erythroid lineage precursors. Collectively, these results continue to suggest an early erythroid/myeloid lineage precursor and/or to the bone marrow stromal niche supporting hematopoiesis as the target of MNX and RDX. These results suggest that MNX- and RDX toxicity in the rat appears to mimic some clinical manifestations of the myeloproliferative disorder, idiopathic myelofibrosis, and thus may offer a model for study of disease progression and intervention strategies.

15. SUBJECT TERMS
hexahydro-1,3,5-trinitro-1,3,5-triazine; hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine; RDX; MNX; anemia; myelosuppression

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INTRODUCTION: The subject of research supported by this grant is a determination of the mechanism through which hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), environmental degradation product of high energetic munition hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), causes anemia. Anemia was detected in our previous acute toxicity studies in the rat (Meyer et al. 2005) and persisted 14 days after a single dose of MNX (NOAEL 47 mg/kg). Since anemia resulting from direct chemical destruction of intravascular erythrocytes is typically resolved within ~7 days in the rat, the 14-day persistence led us to hypothesize that MNX was cytotoxic to erythroid-lineage progenitor cells. Like other myelosuppressive chemicals, we also postulated that longer term, repeat exposure to MNX would be fibrogenic to the bone marrow microenvironment necessary for maturation of hematopoietic stem cells and hence offer an experimental model analogous to human idiopathic myelofibrosis. Further, previous studies on detection of a MNX ring cleavage product suggested this toxicity could be due to metabolism by bone marrow stromal cells. The scope of the proposed work of the overall project encompasses determination of whether: 1) MNX produces persistent bone marrow toxicity, 2) MNX and ring cleavage metabolite MDNA, as produced from metabolism by bone marrow stromal cells, accumulate in bone after acute exposure; 3) acute exposure to MNX produces toxicity to bone marrow progenitors of the erythroid lineage; 4) acute exposure to MNX produces toxicity to the bone marrow stromal microenvironment, and 5) repeated administration of lower doses of MNX produces bone marrow toxicity including fibrosis. In addition, effects of parent RDX on selected endpoints will be assessed to provide structure-activity information relevant to mechanism of hematotoxicity and necessary for assessment of relative risk of the two nitramines in remediation of RDX-contaminated sites.

BODY:

Task 1: Determination of whether acute exposure to MNX produces persistent bone marrow toxicity to include examination of selected endpoints in RDX-exposed rats (Months 1 – 12).

To provide stronger evidence that MNX anemia resulted primarily from effects on bone marrow, we determined whether MNX directly affected peripheral erythrocytes, a potential alternative mechanism for anemia.

An accomplishment relevant to Task 1 is a determination of whether MNX acted like prototypical nitrogenous hematotoxicants, eg. nitrite, in producing anemia secondary to oxidization of hemoglobin Fe2+ to Fe3+ methemoglobin (MtHb). Erythrocytes with precipitated MtHb (ie., Heinz bodies) are then removed from peripheral circulation due to sequestration in splenic red pulp. Two approaches were used to test for MNX oxidation of hemoglobin Fe2+, an *in vitro* incubation of lysed rat erythrocytes with MNX and subsequent colorimetric determination of MtHb and an *in vivo* assessment of MtHb formation in MNX-dosed rats.

Table 1: *in vitro* MtHb study

Compound	Concentration (mM)	Methemoglobin (%)
Vehicle(H_2O)	(n=3)	0.7[a]
$NaNO_2$ (n=2 for all concentrations)	0.013 0.025 0.05	36.8 53.7 71.7
RDX (n=2 for all concentrations)	0.05 0.50 2.5 5.0	1.2 2.3 1.9 3.4
MNX (n=2 for all concentrations)	0.05 0.50 2.5 5.0	0.8 3.7 3.5 1.6

Method, in vitro study: Blood from untreated rats was diluted 1:100 in deionized water. RBC membranes were pelleted by centrifugation (16,000 x g, 15 min) and lysates was incubated for 30 min with varying concentrations of RDX, MNX, TNX or positive control $NaNO_2$. Methemoglobin was then determined as KCN-inhibited absorption (OD 635 nm) as a percentage of that for totally oxidized (K3Fe(CN)6) hemoglobin (Evelyn, K.A. and T. Malloy, 1938).
Results: As evident from data presented in Table 1 above, we have determined that MNX, as well as fully reduced TNX and parent RDX, do not directly redox couple with hemoglobin ferrous iron at concentrations well in excess of blood levels in MNX- or RDX-exposed anemic rats. In contrast, nitrite at a concentration of $1/100^{th}$ that tested for the nitramines oxidizes ~70% of hemoglobin to MtHb.

Method, in vivo study: Female Sprague-Dawley rats (~200 g) were orally gavaged with MNX at 94 mg/kg (LD_{50} ~ 190 mg/kg) or vehicle (5% DMSO in corn oil). Sodium nitrite (40 mg/kg) (n = 3) was used as a positive control. Blood was withdrawn via tail vein at varying times (0m, 30m, 1h, 2h, 4h, 24h, 48h) for MtHb analysis.
Results: As illustrated in Fig 1, right, MtHb after administration of 94 mg/kg MNX, a dose level producing anemia, did not exceed the maximum observed after vehicle (~2.5% at 2 h). In contrast, treatment with $NaNO_2$ at 40 mg/kg gave a marked increase in MtHb at early times (≤ 4 h) that resolved by 24 h. A maximal value of ~45% was observed at 1 h after dosing.

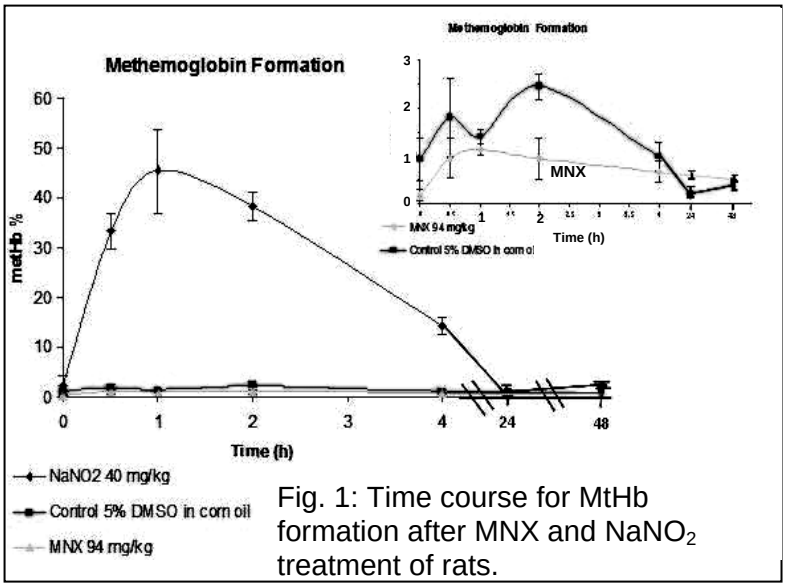

Fig. 1: Time course for MtHb formation after MNX and $NaNO_2$ treatment of rats.

To summarize Task #1 accomplishments, we have provided more substantive evidence supporting the hypothesis that MNX-induced, persistent anemia results from toxic effects of this chemical on hematopoietic stem cells of the bone marrow. A direct evaluation of the ability of MNX to induce anemia by an alternative mechanism, i.e., induction of erythrocyte MtHb formation, was not supported by results of these studies.

Task 2: Determination of whether MNX and metabolite MDNA accumulate in bone after acute exposure and whether bone marrow stromal cells are metabolically capable of converting MNX to MDNA. (Months 13 – 36).

A manuscript describing previous collaborative work with U.S. Army Corps of Engineers colleagues (MacMillan, Denise, et al.) on disposition and metabolism of RDX and MNX has been accepted for publication in *Toxicol. Mechanisms and Methods.* The Meyer lab contribution to this project was funded through other DoD support (DACA42-02-P-0035).

No further independent progress by the Meyer lab has been made on identifying bone marrow MNX and metabolites. Dr. MacMillian has left our collaborators' laboratory at USACE in Vicksburg, MS; however, we are currently preparing a contract for pursuit of these studies

with Dr. Tony Bednar who is continuing Dr. MacMillian's work. ULM graduate student, Mitchell Wilbanks, will be conducting these studies.

Task 3: Determination of whether acute exposure to MNX produces toxicity to bone marrow progenitors of the erythroid and myeloid lineages. (Months 18 – 36)

Accomplishments relevant to Task 3 include a continuation of the year 1 study determining effects of RDX and MNX on colony forming activity of the erythroid (BFU-E) and myeloid (CFU-GM) lineages. These studies have been expanded from year 01 studies, which characterized effects after 14 d post treatment, to include an assessment at 7 d. In addition, effects of RDX and MNX on the bone marrow stem cell immediately proximal to the split of the myeloid and erythroid pathways, i.e., the CFU-GEMM, has been evaluated at 7d. Finally, the effects of the nitramines on the relative populations of myeloid and erythroid lineages has been assessed by flow cytometric analysis of bone marrow cells from treated rats at 14 d.

Method: Bone marrow cells were flushed from femurs into ice-cold Iscoves Modified Dulbecco Medium (IMDM) plus 2 % FBS and antibiotic/antimycotic. Cells were washed (200 x g, 10 min) and mononuclear cells isolated using Histopaque-1077^{TM} (Sigma Chemical Co, USA) according to supplier's protocol. Separated mononuclear cells were counted using a hemocytometer. Progenitor cells of the myeloid (CFU-GM) and erythroid (BFU-E) lineages and a common erythroid/myeloid progenitor cell (CFU-GEMM) were assayed using Halo kits as per supplier's instruction (Hemogenix Inc, USA). In brief, 20,000 mononuclear cells were treated with growth factors (GM-CSF, IL-3, SCF for CFU-GM; EPO, IL-3, SCF for BFU-E; and EPO, GM-CSF, G-CSF, IL-3, IL-6, SCF, TPO, Flt3-L for CFU-GEMM), methyl cellulose, serum and incubated in 96-well plates in a CO_2 incubator for 5 days. After 5 days, colony forming activity was monitored in a high through-put system in which cell numbers were deduced from fluorescent measurements of amount of ATP per well. Amount of ATP was quantified against the standard curve generated on the same day.

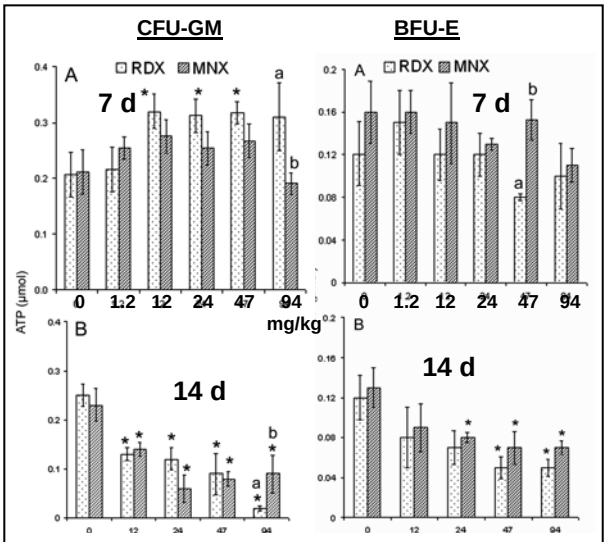

Fig. 2: Myeloid and Erythroid lineage assays of bone marrow cells.

Results: The top panels of Figure 2 illustrates the effects of RDX and MNX on colony forming ability of myeloid (CFU-GM) and erythroid (BFU-E) progenitor cells 7 d after treatment. Neither RDX (open bars) nor MNX (filled bars) decreased CFU-GMs or BFU-Es as had been noted in the previous study at 14 d post-treatment (Fig 2; bottom panels). In contrast, there was stimulation of CFU-GMs by RDX, but not MNX, at 7 d after treatment with doses of ≥ 12 mg/kg.

Myeloid/erythroid precursor cells (CFU-GEMMs) were likewise not decreased 7 d after nitramine treatment (Fig. 3). In this case, increase colony forming activity was observed at intermediate doses of RDX (47 mg/kg) and MNX (1.2 and 12 mg/kg).

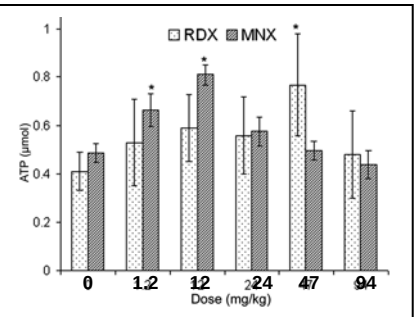

Fig. 3: Assay for common myeloid/erythroid progenitor bone marrow cells.

Flow cytometry studies were also performed to assess the relative developmental state of the erythroid lineage cells targeted by the nitramines. These studies utilize flow cytometric assessment of frequency of cells expressing early myeloid (Thy1.1) and later erythroid (CD71; transferrin receptor) lineage markers at 14 d after MNX exposure.

Method: Total bone marrow cells (CD71 protocol) and mononucleated bone marrow cells (Thy 1.1) were obtained as described above and incubated at 5 x 10^6 cells/ml in phosphate buffered saline (PBS) containing 0.1 % sodium azide. The cell suspension (200 µl) was kept on ice in a 15 ml conical centrifuge tube. Low affinity Fc receptors were blocked with mouse anti-rat CD32 [FcγIII/II, 1: 2000, 10 min]. The cells were then incubated with primary antibodies: PE-conjugated antibody to rat Thy1.1 (1:2000) and FITC-conjugated mouse monoclonal antibody to rat CD71 (1: 200) for 45 min on ice in the dark. Following incubations, cells were then washed twice with 1 ml PBS (400 x g, 4°C, 10 min) then cells were re-suspended in 500 µl PBS. Aliquots containing only single primary antibody (for fluorescence spill over compensation) and isotype antibodies (negative control) were processed simultaneously for each experiment. A BD FACSCaliber flow cytometer (BD Biosciences, San Jose, CA) was setup using isotype control. Fluorescence spillover was nullified by compensation using individually labeled cells for primary antibodies with each experiment. FITC fluorescence was measured on the FL1 (band pass filter, 530 ± 15 nm) detector whereas PE fluorescence was measured on the FL2 (band pass filter, 585 ± 21 nm) detector. Data was collected using linear (forward and side scatter) and logarithmic (FL1 and FL2) scales. For each sample data from 20,000 cells were acquired with low flow rate (12 µl). Data were analyzed using Cell Quest Software (BD Biosciences, San Jose, CA). Region markers for M:E ratio analysis were set according to earlier report (Schomaker, Clemo, and Amacher 2002). Antibodies were mouse anti-rat CD32 (Rat Fc block [FcγIII/II], mAB D-34-485, phycoerythrin (PE)-conjugated IgG_1 κ-isotype control antibody, fluorescein isothiocyanate (FITC)-conjugated IgG_{2a} κ-isotype control antibody, PE-conjugated antibody to rat Thy1.1 (mAB; OX-7) and FITC-conjugated mouse monoclonal antibody to rat CD71 (mAB OX-26) from BD Pharmingen (San Jose, CA).

Results: Results from these studies are shown in Fig 4 below and quantitated in Table 2. Consistent with the colony forming unit assays, these data did not indicate that one leg of the myeloid or erythroid pathway was differentially affected by MNX.

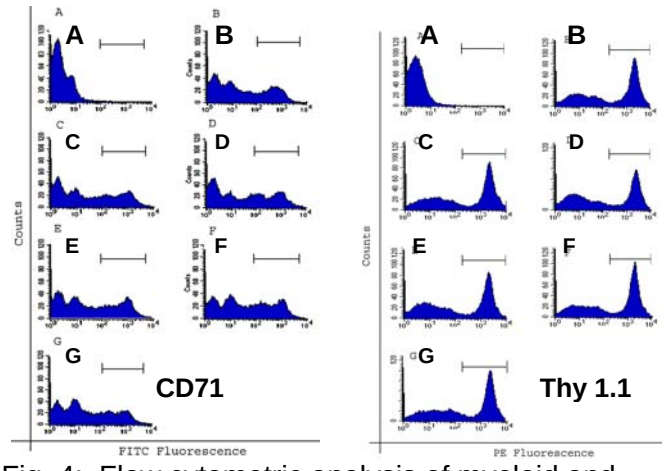

Fig. 4: Flow cytometric analysis of myeloid and erythroid precursors 14 d after MNX. Region markers are indicated by the horizontal bar in each panel.

Table 2: Quantitative flow cytometry data

Dose (mg/kg)	% CD71 expression	% Thy1.1 expression
0 [B]	28.8 ± 3.1	50.8 ± 3.1
1.2 [C]	28.2 ± 2.5	53.2 ± 0.9
12 {D]	27.1 ± 3.2	46.7 ± 1.7
24 [E]	27.6 ± 2.0	50.9 ± 6.2
47 [F]	25.5 ± 3.1	56.3 ± 0.4
94 {G]	26.7 ± 2.0	56.8 ± 3.0

It should also be noted hematology was also done on the 7d post treatment studies and no effect of MNX or RDX was seen on blood hemoglobin or hematocrit in contrast with the decreases observed at 14 d post treatment.

Accomplishments under Task #3 have directly addressed RDX and MNX effects on specific bone marrow progenitor cells and studies have provided data consistent with the hypothesis that these compounds are targeting hematopoietic stem cell development in the bone marrow. These newer studies unequivocally demonstrate that the target cells adversely affected by MNX and RDX are stem cells more proximal in the hematopoietic development pathway than the bifurcation point of commitment towards either the myeloid or erythroid pathways. Alternately, the supportive environment of the stem cell niche may be compromised. The latter will be addressed in future Task #4 and #5 studies.

Task 4: Determination of whether acute exposure to MNX produces toxicity to the bone marrow stromal microenvironment. (mos. 12 – 30)

No progress in yr 02.

Task 5: Determination of whether repeated administration of lower doses of MNX produces bone marrow toxicity, especially fibrosis. (mos. 24 – 36).

Previous graduate student Melissa Aycock, had conducted pilot studies to optimize histopathological techniques for bone in preparation of conducting repeat dose studies to detect bone marrow fibrosis. Ms. Aycock was dismissed from the ULM graduate program for academic reasons. Since, another graduate student. Madhura Rane, continued the project, but left after 1 yr for personal reasons. A third student, Sindhura Ramasahayam, has been hired and has recently begun where the previous students left off. Collection of bone marrow samples from MNX exposures for determination of fibrogenic growth factors by immunoassay has been completed.

Statistics: Effects of MNX and RDX on hematological parameters were determined by ANOVA with post-hoc comparisons of treatment means against vehicle control done with Dunnett's test and difference between MNX and RDX was assessed with a student's *t*-test. Results were considered statistically significant with $p < 0.05$. Data were statistically analyzed using JMP 4.0.4 software (SAS Institute Inc.).

KEY RESEARCH ACCOMPLISHMENTS:

Studies thus far have demonstrated:

- support for the hypothesis that MNX-induced, persistent anemia results from toxic effects of this chemical on hematopoietic stem cells of the bone marrow.
- suggested that a common up-stream multipotential stem cell of both the myeloid and erythroid lineages and/or the stromal microenvironment as a target.
- determined that the parent chemical RDX of environmentally produced MNX also has similar effects on this multipotential stem cell of both the myeloid and erythroid lineages and/or the stromal microenvironment.

REPORTABLE OUTCOMES:

1. Abstract accepted for presentation at 2008 annual meeting of the Society of Toxicology, Charlotte, NC

Wilbanks, M.S., S.L. Reeves, L.S. Inouye, E.J. Perkins, B.L. Escalon and S.A. Meyer. Comparative Hematotoxicity of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) vs. Mono- and Tri-Nitroso Environmental Degradation Products MNX and TNX. *The Toxicologist. 2008; 921 (S-1): in press.*

CONCLUSION: Results of Yr 02 studies continue to support the hypothesis that hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), environmental degradation product of high energetic munition hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), is toxic to hematopoietic bone marrow stem cells. Data suggest toxicity to an early erythroid/myeloid lineage precursor and/or to the bone marrow stromal niche supporting hematopoiesis. Thus, previously observed peripheral blood disorders in MNX-treated rats appear to be the consequence, in part, of loss replenishing stem cell populations. In addition, the delay in manifestation of myelosuppression is interpreted as resulting from the interval between occurrence of adverse effect on an early stem cell and expression in later committed bone marrow cells and peripheral blood. As such, this system appears to mimic some clinical manifestations of the myeloproliferative disorder, idiopathic myelofibrosis, and thus offer a model for study of possible mechanisms of disease progression and development of intervention strategies.

Additionally, we are able to conclude that the parent RDX, like MNX, causes myelosuppression. In addition to the adverse hematological effects we have documented, these results suggest additional functional consequences with respect to host resistance to infection, inflammation and tissue trauma. As relates to the role of the Department of Defense in remediation of RDX-contaminated sites, **collectively these data argue that risk of adverse hematological effects from exposure are lessened upon natural remediation to nitro reduced products**.

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APPENDICES: None

SUPPORTING DATA: None